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Involvement of ERK, p38 MAP Kinase, and PKC in MHC Class II-Mediated Signal Transduction in a Resting B Cell Line

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Substantial evidence suggests that MHC class II molecules play a critical role in transducing signals during B cell activation and differentiation. In addition, we previously found that cross-linking of MHC class II molecules using anti-MHC class II antibodies inhibited NF-κB activation in resting B cells isolated from mouse spleen. In this study, we investigated the mechanism of anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation using a resting B cell line, 38B9. We found that treatment with a corresponding anti-MHC class II antibody reduced the activation of NF-κB in LPS-stimulated 38B9 cells, treatment of the antibody mediated down-regulation of PKC and ERK/p38 MAP kinase pathways, and treatment with PKC inhibitors caused down-regulation of ERK and p38 MAP kinase activities in LPS-stimulated 38B9 cells. Our results suggest that the PKC and ERK/p38 MAP kinase pathways are regulated by anti-MHC class II antibodies, and that MHC class II molecules are actively involved in the signal transduction pathway in the resting B cell line, 38B9. Consequently, disruption of these pathways might contribute to the inhibition of LPS-induced NF-κB activation in 38B9 cells.

Key Words: **B lymphocyte; major histocompatibility complex; signal transduction.**

Development of B cells from progenitors into terminally differentiated plasma cells is a multistep process involving signal transduction and ordered expression of a large number of genes. Surface molecules of B cells, including MHC molecules, play an essential role in transducing signals for B cell activation and differentiation and regulation of the related immune responses (1, 2). MHC class II molecules are reportedly capable of transducing signals related to B and T cell activation (3–6). In B cell activation, signal transduction through MHC class II molecules is known to occur via two distinct mechanisms, dependent on their state of differentiation. In resting B cells, signal transduction involves the generation of cAMP and the localization of PKC in the cytoskeletal/nuclear compartment (3, 6, 7). In primed B cells, signal transduction involves tyrosine kinase activation, inositol lipid hydrolysis, and Ca^{2+} mobilization. However, these events are only coupled in cells activated by a specific stimulus (4, 5, 8). We previously reported that cross-linking of MHC class II molecules with anti-MHC class II antibodies and dimeric epitope peptides inhibited NF-κB activation in resting B cells isolated from mouse spleen (9). Based on these findings, we assumed that induction of signal transduction events through MHC class II molecules leads to the activation of transcription factors, such as NF-κB and AP-1, resulting in the expression of genes required for B cell activation and differentiation (10, 11). In addition, the results that anti-MHC class II antibodies inhibit B cell proliferation and *in vitro* antibody production by B cells support our assumption (12–14). However, the process involved in MHC class II-related regulation of mitogen-induced B cell proliferation and differentiation accompanying NF-κB activation is not clear. In an effort to understand the mechanism involved in multilateral B cell activation, we investigated the influence of anti-MHC class II antibody treatment on LPS-induced activation of NF-κB and the consequent signaling pathways using a resting B cell line, 38B9. We demonstrated that anti-MHC class II antibody treatment suppressed LPS-induced activation of NF-κB, and that this suppression was mediated by the sequential down-regulation of PKC and ERK/p38 MAP kinase activity induced by LPS.

Abbreviations used: MHC, major histocompatibility complex; PKC, protein kinase C; ROS, reactive oxygen species.

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MATERIALS AND METHODS

Materials. The chemicals and plastics used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Becton–Dickinson, Franklin Lakes, NJ), respectively (unless specified). Antibodies specific to ERK and p38 MAP kinase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to I-A^k and I-A^d were purified from the culture supernatants of 10-3.6.2 and MK-D6 hybridoma cells, respectively (15–17).

The resting B cell line, 38B9 (18) originated from BALB/c mice (*d*-haplotype), was provided by Dr. G.-H. Chung (Chonbuk National University). The cell line was cultured in RPMI 1640 medium supplemented with 5% FBS (HyClone, Logan, UT) and 50 μM 2-mercaptoethanol.

Western blot analysis. 38B9 cells treated with 50 μg/ml of LPS and anti-MHC class II antibody or PKC inhibitors were lysed with lysis buffer (150 mM NaCl, 1% NP-40, 125 mM PMSF, 50 mM Tris–HCl, pH 7.4). Supernatants from the cell lysates were immunoprecipitated with anti-ERK (sc-94) or anti-p38 (sc-728) antibodies, pre-absorbed with protein A-Sepharose 4B. The immunoprecipitates were resolved by 12% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. Western blot analyses were performed using anti-p-ERK (sc-7383) or anti-p-p38 (sc-7973) antibodies followed by incubation with a FITC-conjugated secondary antibody. Signals were analyzed using a Fuji LAS1000 imager (Fuji Photo Film Co., Japan).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared using 0.5–1 $\times$ 10^7 38B9 cells as previously described, and protein concentrations were determined using the Bradford reagent (19, 20). To measure the extent of NF-κB activation, EMSA was carried out at room temperature for 30 min in a total volume of 20 μl, containing 10 μg nuclear extract proteins, 500 ng of poly(dI-dC) as a nonspecific competitor, and 4 ng of ^{32}P-labeled double stranded NF-κB consensus oligonucleotides (5′-CCGCTTAACAGAGGGGGCTTTCCGAG-3′). The DNA–protein complex was resolved using 4% native polyacrylamide gel electrophoresis.

Protein kinase C assay. To measure the PKC activity, cells were lysed with an extraction buffer (0.05% Triton X-100, 10 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.5 mM EDTA, 25 mM Tris–HCl, pH 7.4). The supernatant was applied to a DEAE-cellulose column preequilibrated with the extraction buffer. The PKC-containing fraction was eluted using an extraction buffer containing 200 mM NaCl (21). Protein concentrations were determined using the Bradford reagent (19). PKC activity was determined using the PepTaq Non-Radioactive Protein Kinase C assay kit (Promega, Madison, WI), in which a fluorescent PKC-specific peptide (P-L-S-R-T-L-S-V-A-A-K) was used as a substrate. To quantify PKC activity, the negative-charged phosphorylated bands were excised from the gel and heated at 95°C. The absorbance of the gel solution was read at 570 and 480 nm and units of PKC activity were calculated. One unit of PKC activity was defined as the number of nanomoles of phosphate transferred to a substrate per minute per milliliter.

RESULTS

Influence of Anti-MHC Class II Antibody Treatment on LPS-Induced NF-κB Activation

NF-κB, a nuclear transcription factor, is associated with regulating the gene expression of cytokines (such as IL-6) and various regulatory genes required for the activation, development, and continued survival of lymphocytes (22, 23). To confirm the effect of anti-MHC class II antibody treatment on the level of LPS-induced NF-κB activation in resting B cell line, 38B9, LPS-stimulated 38B9 cells were treated with an anti-MHC class II antibody. Nuclear extracts prepared from the cells were used to determine NF-κB activation by EMSA (Fig. 1). LPS (50 μg/ml) treatment was found to increase the expression of activated NF-κB in 38B9 cells. However, 50 μg/ml of anti-I-A^d antibodies, which were specific to the MHC class II molecules of 38B9 cells, reduced the activation of NF-κB in LPS-stimulated 38B9 cells. Conversely, the same amount of unrelated anti-I-A^k antibodies did not reduce the activation of NF-κB in LPS-stimulated 38B9 cells. This result demonstrated that cross-linking of MHC class II molecules with an anti-MHC class II antibody was involved in the reduction of LPS-induced NF-κB activation in 38B9 cells.

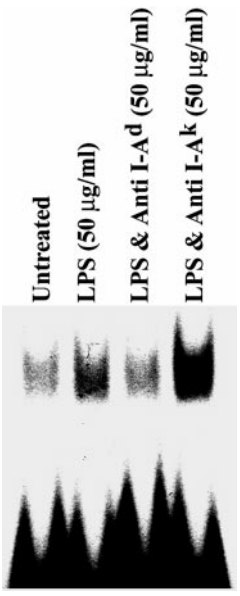

FIG. 1. Influence of anti-MHC class II antibody treatment on NF-κB activation of LPS-stimulated 38B9 cells. Equal numbers of cells were treated for 30 min as described and later disrupted by hypotonic lysis in the presence of protease inhibitors. After complete lysis, intact nuclei were harvested by centrifugation. High salt extracts of nuclei were prepared and tested by EMSA using a NF-κB probe, as described under Materials and Methods. After the binding reaction for 20 min, the mixture was electrophoresed through a native 4% polyacrylamide gel. The gel was dried and an autoradiogram was obtained after overnight exposure at −70°C.

Influence of Anti-MHC Class II Antibody Treatment on the LPS-Induced Activation of ERK and p38 MAP Kinase

Several distinct signaling pathways regulate the activation of NF-κB (24, 25). Western blot analysis was used to assess the involvement of ERK and p38 MAP kinase in anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation in 38B9 cells (Fig. 2). We first determined whether the activation of ERK and p38 MAP kinase was necessary for the LPS-

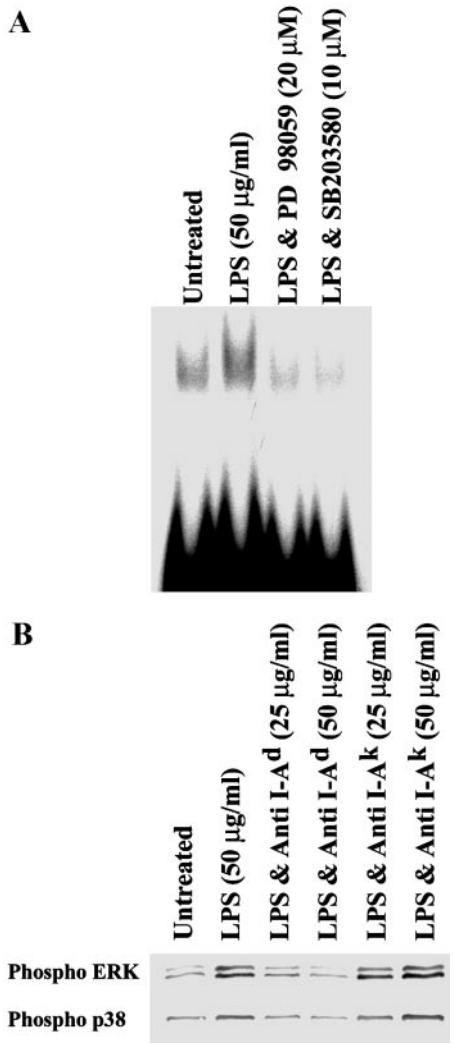

FIG. 2. Influence of anti-MHC class II antibody treatment on the activation of ERK and p38 MAP kinase in LPS-stimulated 38B9 cells. (A) Effect of ERK and p38 MAP kinase inhibitors, PD 98059 and SB 203580, respectively, on LPS-induced NF-κB activation in 38B9 cells. To identify NF-κB activation, EMSA was performed as described in the legend to Fig. 1. (B) Level of LPS-induced ERK and p38 MAP kinase activation in 38B9 cells after treatment with anti-MHC class II antibodies. Total cellular extracts were prepared from equal numbers of cells treated as previously described. The cellular extracts (100 μg/sample) were incubated with either anti-ERK or anti-p38 antibody, and then with A-Sepharose protein. The resin was harvested by centrifugation and eluted with SDS–PAGE sample buffer. Eluted proteins were separated by SDS–PAGE on 12% gels, and electroblotted onto nitrocellulose membranes. Blots were probed with the primary antibody, either anti-phospho-ERK or anti-phospho-p38 antibody, followed by a FITC-conjugated goat anti-mouse IgG secondary antibody. The signals were analyzed using a Fuji LAS 1000 Image Analyzer.

triggered signaling pathway that leads to NF-κB activation in 38B9 cells (Fig. 2A). As shown in the figure, inhibition of ERK and p38 MAP kinase by the selective inhibitors PD 98059 (20 μM) and SB 203580 (10 μM), respectively (26, 27), suppressed the LPS-induced NF-κB activation. This indicated that activation of ERK and p38 MAP kinase was required for the activation of NF-κB in LPS-stimulated 38B9 cells.

The effect of the cross-linking of MHC class II molecules on ERK/p38 MAP kinase activation was then assessed (Fig. 2B). LPS treatment of cells increased both the level of phosphorylated ERK and phosphorylated p38 MAP kinase. However, treatment of LPS-stimulated 38B9 cells with the corresponding anti-I-A^d antibody, but not with the anti-I-A^k antibody, reduced the level of phosphorylation of ERK and p38 MAP kinase in a dose-dependent manner. The total ERK and p38 MAP kinase protein levels expressed were not affected by treatment with anti-MHC class II antibody (data not shown), suggesting that the anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation was linked with both ERK- and p38 MAP kinase-associated pathways.

Influence of Anti-MHC class II Antibody Treatment on the LPS-Induced PKC Activation

Protein kinase C (PKC) is reported to play a pivotal role in the regulation of B cell differentiation induced by mitogens, such as LPS and phorbol esters (28, 29). Therefore, to determine if PKC, which may participate in other signal transduction pathways that promote LPS-induced NF-κB activation, is related to the anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation in resting B cells, modulators of PKC activity were administered to cells and the effects on NF-κB activation were examined (Fig. 3). The PKC inhibitors, chelerythrine and H-7 (30, 31), were administered to 38B9 cells to confirm that a PKC-associated signaling pathway mediated the LPS-induced NF-κB activation (Fig. 3A). The figure illustrates that treatment of 38B9 cells with PKC inhibitors reduced the LPS-induced NF-κB activation. This suggests that PKC is involved in a signaling pathway linked to LPS-induced NF-κB activation in 38B9 cells.

Cross-linking of MHC class II molecules with anti-MHC class II antibody was investigated in order to determine whether it was associated with a LPS-triggered signaling pathway leading to the activation of NF-κB via PKC activation. Phorbol 12,13-dibutyrate (PDBU) was used as a PKC activator, since phorbol esters reportedly work as PKC activators and induce NF-κB activation in many cell lines (32–34). As shown in Fig. 3B, NF-κB activation was induced by PDBU treatment of cells (0.2 μM). In addition, 50 μg/ml of the corresponding anti-I-A^d antibody inhibited the PDBU-mediated NF-κB activation. However, anti-I-A^k antibody, which is unrelated to the MHC molecules on 38B9 cells, did not inhibit PDBU-induced NF-κB activation. These results suggest that PKC is involved in the signaling pathway associated with the anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation in the resting B cell line.

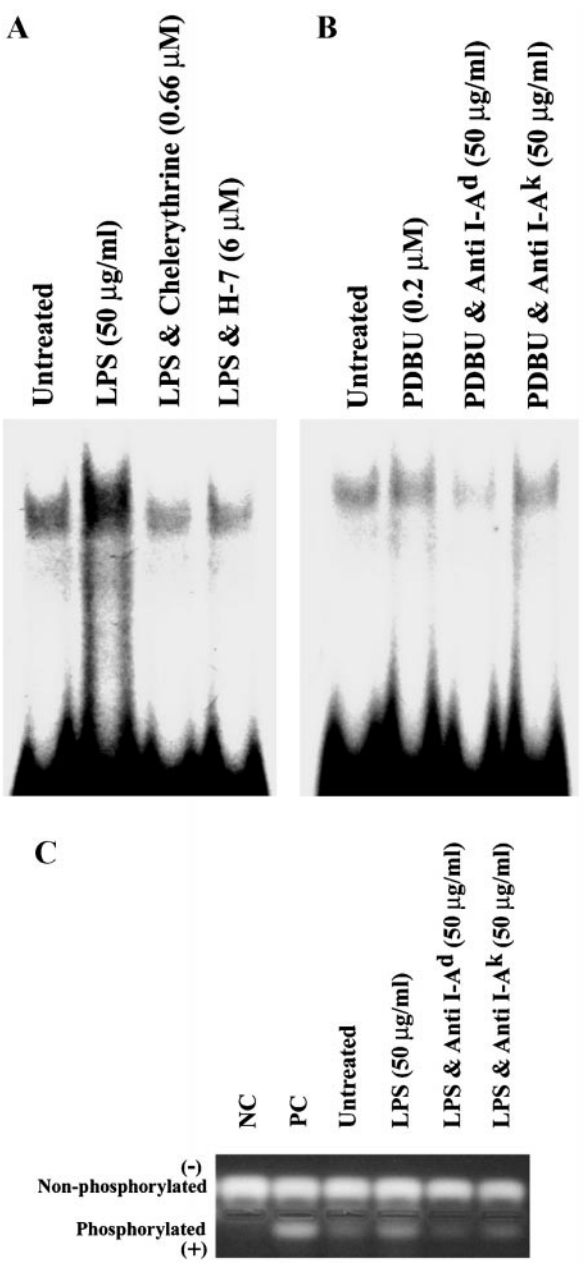

FIG. 3. Influence of anti-MHC class II antibody treatment on the PKC activation by LPS-stimulated 38B9 cells. Effects of (A) PKC inhibitors on the LPS-induced NF-κB activation, and (B) anti-MHC class II antibodies on the PDBU-induced NF-κB activation in 38B9 cells. Equal numbers of cells were treated as previously described for 30 min and then disrupted by hypotonic lysis in the presence of protease inhibitors. To identify NF-κB activation, EMSA was performed as previously described in the legend to Fig. 1. (C) Effect of anti-MHC class II antibody treatment on LPS-induced PKC activity in 38B9 cells. One microgram of PKC-specific peptide substrate was incubated without (NC) or with 5 ng of PKC (PC), or with 3.3 μg of partially purified samples prepared from cells that were treated as previously described. After reacting for 30 min at room temperature, reactions were stopped by heating to 95°C for 10 min. Samples were electrophoresed on a 0.8% agarose gel at 100 V for 15 min. Phosphorylated peptides migrated toward the anode (+), and nonphosphorylated peptides migrated toward the cathode (−). The gel was photographed on a transilluminator.

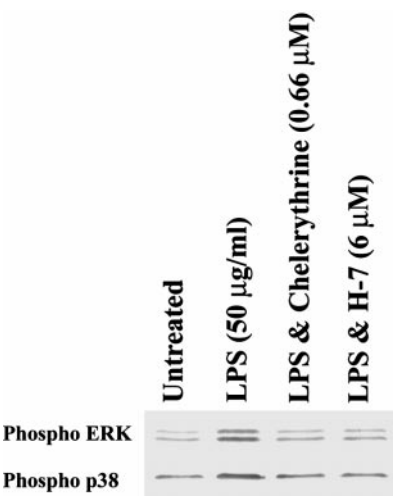

FIG. 4. Influence of PKC inhibitor treatments on the LPS-induced activation of ERK and p38 MAP kinase. Total cellular extracts were prepared from equal numbers of cells treated as previously described. The ERK and p38 MAP kinase activation assay was performed as described in the legend to Fig. 2.

Using a PKC assay kit, the involvement of PKC in the anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation in 38B9 cells was confirmed (Fig. 3C). The level of activated PKC was represented by the extent of migrated phosphorylated substrates of PKC. As shown in the figure, the calculated PKC activity (0.6 U) of the sample prepared from the LPS-treated 38B9 cells was up to three times higher than that (0.2 U) prepared from the untreated cells. When 38B9 cells were treated with LPS and anti-I-A^d antibody together, PKC activity was calculated to be 0.4 U representing approximately two thirds that of control cells treated with LPS alone. However, calculated PKC activity of the sample prepared from the 38B9 cells treated with same amount of LPS and antibody specific to the unrelated I-A^k molecules was 0.6 U suggesting that only the related antibody was effective for the inhibition of LPS-induced PKC activity in 38B9 cells. Collectively, these results suggest that anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation in 38B9 cells is dependent on PKC activity.

Influence of PKC Inhibitor Treatment on the LPS-Induced Activation of ERK and p38 MAP Kinase

Previous results indicated that LPS-induced activation of NF-κB required activation of both PKC and ERK/p38 MAP kinase. Consequently, the effect of PKC inhibitor treatment on the LPS-induced activation of ERK and p38 MAP kinase was investigated, and vice versa. The PKC inhibitors, chelerythrine and H-7, down-regulated the LPS-induced activation of ERK and p38 MAP kinase (Fig. 4). Conversely, PKC activity

was not significantly inhibited by ERK (PD 98059) or p38 MAP kinase (SB 203580) inhibitors in LPS-stimulated 38B9 cells (data not shown). These results indicate that LPS-induced PKC activation results in the activation of ERK and p38 MAP kinase.

DISCUSSION

Various signaling pathways involving MHC class II molecules regulate B cell activation and differentiation (3, 35). NF-κB is considered to be one of the crucial transcription factors involved in signal transduction from the cytoplasm to the nucleus. Previously, we investigated whether MHC class II molecules are associated with the activation of NF-κB in LPS-stimulated resting B cell populations isolated from mouse spleen. Cross-linking of MHC class II molecules was demonstrated to inhibit LPS-induced NF-κB activation in resting B cells (9). In the present study, MHC class II molecules were found to be similarly associated with the activation of NF-κB in the LPS-stimulated resting B cell line, 38B9 (Fig. 1); the corresponding anti-MHC class II antibody reduced the activation of NF-κB in the LPS-stimulated 38B9 cells. Treatment of LPS-stimulated 38B9 cells with anti-MHC class II antibody also inhibited the production of IgG and expression of the cytokine IL-6, a critical cytokine involved in B cell differentiation (data not shown). Anti-MHC class II antibody-mediated inhibition of NF-κB activation might not be due to the nonspecific or Fc receptor-mediated signals because treatment of the cells with unrelated- and isotype matched-antibodies did not show any inhibitory effect on NF-κB activation. Prepared $F(ab')_2$ fragments of anti-MHC class II antibodies were tested for their ability to inhibit the production of secreted IgG (data not shown). As expected, whole molecules and $F(ab')_2$ fragments of the corresponding anti-MHC class II antibodies showed a similar inhibitory effect on LPS-stimulated 38B9 cells (data not shown). Interestingly, $F(ab')_2$ fragments of unrelated anti-MHC class II antibodies showed weak activation of the production of secreted IgG compared to whole molecules. These findings suggest that this inhibitory response does not involve Fc receptors and that cross-linking of MHC class II molecules is essential to causing this inhibitory response. However, there is a possibility that Fc receptor binding to unrelated anti-MHC class II antibodies in B cells might contribute to up-regulation of LPS-induced production of secreted IgG. In addition, the possibility that the inhibitory effects triggered by anti-MHC class II antibodies are due to the induction of apoptosis could also be excluded by the observation that DNA laddering did not appear in response to the treatment with anti-MHC class II antibody for 24 h (data not shown). Thus, the anti-MHC class II antibody-mediated inhibition of LPS-induced activation of NF-κB in resting B cells appeared to be directly related to its specificity for MHC class II molecules.

Resting B cells can be activated by various mitogens, including LPS, for proliferation and subsequent differentiation into antibody-secreting cells. LPS also triggers a cascade of intracellular signaling pathways, including those activating PTK, PKC, and PKA. This study attempted to understand the mechanism(s) by which LPS-induced NF-κB activation is inhibited by the anti-MHC class II antibody by examining the effect of anti-MHC class II antibody treatment on various signaling pathways involved in the activation of NF-κB. Of the molecules involved in the cascade of intracellular signaling pathways (36), two down-stream kinases, ERK and p39 MAPK involved in the MAP kinase cascades, were first examined. Mitogen-activated protein kinases are important cellular signaling components that convert various extracellular signals into intracellular responses (37). In mammalian cells, three distinct MAP kinase cascades have been identified (38). These include extracellular signal-regulated protein kinase (ERK), c-Jun-N-terminal kinase (JNK/SAPK), and p38 MAP kinase. We found that anti-MHC class II antibody treatment of 38B9 cells reduced the level of ERK and p38 MAP kinase phosphorylation triggered by LPS, in a haplotype-specific, dose-dependent manner (Fig. 2). However, no phosphorylated JNK was detected after the treatment of anti-MHC class II antibody although the positive control produced clear band (data not shown), suggesting that JNK was not involved in this event. Next, PKC involvement in the inhibition of LPS-induced NF-κB activation by the anti-MHC class II antibody was investigated. As shown in Fig. 3, PKC inhibitors inhibited LPS-induced activation of NF-κB and treatment with the corresponding anti-MHC class II antibody reduced the activation of NF-κB triggered by the PKC activator PDBU. These observations, along with the fact that anti-MHC class II antibody treatment inhibited PKC activity in LPS-stimulated 38B9 cells (Fig. 3C), suggest that PKC and ERK/p38 MAP kinase are involved in the anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation in 38B9 cells. Finally, the fact that some members of the MAP kinase family are regulated by PKC activation prompted an assessment of whether activation of the ERK and p38 MAPK pathways is also regulated by PKC in LPS-stimulated 38B9 cells. The fact that LPS-induced activation of ERK and p38 MAPK in 38B9 cells was inhibited by PKC inhibitors suggested that LPS-induced activation of the ERK and p38 MAP kinase pathways was dependent on PKC activation in 38B9 cells (Fig. 4). Taken together, it is highly plausible that LPS-induced NF-κB activation in 38B9 cells is mediated by sequential activation of PKC and ERK/p38 MAP kinase in that PKC activated by LPS phosphorylates ERK/p38

MAP kinase resulting in its activation, leading to activation of NF-κB.

In conclusion, our finding that down-regulation of the LPS-induced activation of the PKC-dependent ERK/p38 MAP kinase pathway is associated with an anti-MHC class II antibody-mediated inhibition of LPS-induced NF-κB activation in the resting B cell line, 38B9, demonstrated that MHC class II molecules located between LPS stimulation and PKC activation played a critical role in signal transduction during LPS-induced B cell activation and differentiation. We assume that inhibition of the LPS induced NF-κB activation by the cross-linking of MHC class II molecules could be understood as the prior occupation of secondary messengers shared with other receptors or the consequence of an upstream inhibition of signaling via the LPS receptor complex. Therefore, we are currently investigating how the expression of upstream signaling molecules is involved in the anti-MHC class II antibody-mediated inhibition of the LPS-induced NF-κB activation.

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